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Note

A newly discovered baculovirus induces reflex bleeding in the butterfly *Heliconius himera* (Nymphalidae: Heliconiinae)

Heliconius himera (Nymphalidae: Heliconiinae) and other members of the subfamily Heliconiinae constitute some of the most frequently purchased organisms for butterfly exhibitions. Detailed life table data, including information on affiliated diseases, is lacking for many butterflies including *H. himera*. During the summer 2002, a greenhouse colony of *H. himera* in its fifth generation, began to show signs of a disease that gradually decimated the colony. At this time, larvae possessed abnormally thickened, bent cuticular spines. Incidental contact of these larvae with forceps or needles and/or with conspecifics stimulated a pronounced reflex bleeding (Cuernot, 1896) from the cuticular spines in sick larvae (Fig. 1). When mechanical stimulation ceased, a portion of the exuded droplets was siphoned back into the spines. It should be noted that reflex bleeding is not observed in healthy larvae. Throughout the progression of the disease, *H. himera* larvae displayed this reflex bleeding with emitted drops often being deposited on leaves. During the final stages of the disease, the larvae moved very slowly and ceased feeding. Immobile larvae remained alive for two to nine days. Few *H. himera* larvae reached pupation, and when this did occur, the pupae dried out and turned black.

Light microscopy of the exudate revealed the presence of numerous polyhedral-shaped occlusion bodies (OBs) in the emitted fluid. In addition to the OBs, the fluid also contained intact cells (hemocytes), suggesting that the exudate was hemolymph and not a glandular secretion. Light-microscope examination of tissues dissected from diseased larvae revealed the presence of OBs in the nuclei. Fluid released from the tips of the spines, determined to contain numerous OBs, was subjected to sucrose gradient (30–60% w/w) centrifugation. The band containing OBs was washed by two cycles of low-speed centrifugation, diluted in water to produce concentrations of 10^6, 10^5, 10^4 OBs/ml, and painted onto *Passiflora biflora* leaves ($\sim$1 cm^2). Groups of five second-instar larvae were placed individually on treated or control (H_2O) leaves and allowed to feed for 24 h at 26 °C. All control insects developed to the pupal stage. Larvae exposed to leaves coated with 10^6, 10^5, and 10^4 OBs/ml

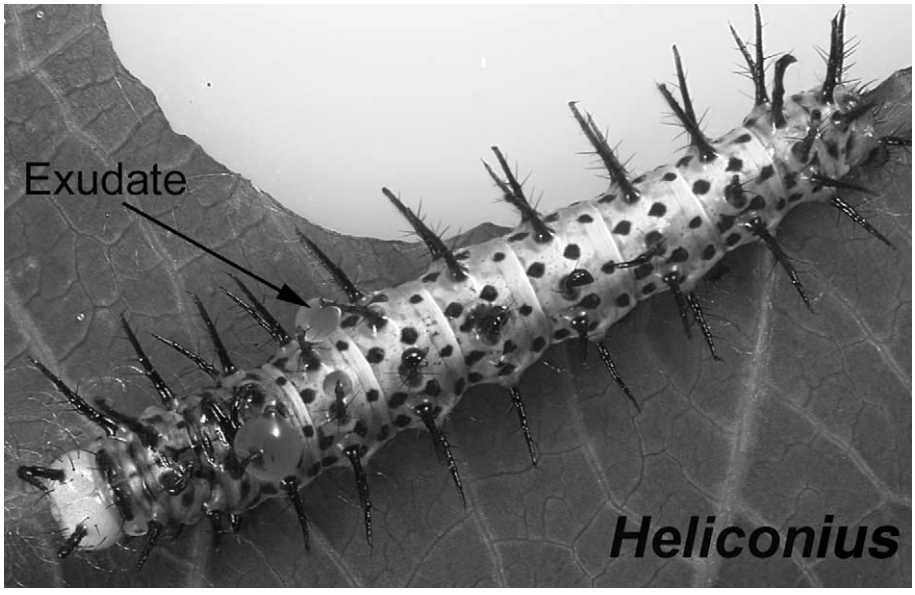

Fig. 1. Baculovirus infected fourth instar *H. himera* that has been agitated with dissecting needle. Reflex bleeding produces droplets at the tips of the cuticular spines. The spines show blunt-ended or bent appearance.

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showed initial signs of the viral infection at the onset of the third instar. Reflex bleeding was copious upon mechanical stimulation and/or vibration of the aquarium. Thirteen of the treated larvae died at the third or fourth instar. Two larvae completed the fourth-instar, which lasted 3 days and reached adulthood. During the final stages of the disease, the larvae moved very slowly and ceased feeding. Immobile larvae remained alive for 2–9 days.

Spines and underlying tissues and exudate samples from infected larvae were dissected, fixed, dehydrated, cleared and infiltrated with Epon–Araldyte resin. Thin sections stained with uranyl acetate followed by Reynolds lead citrate were examined under an electron microscope. Electron microscopy of both fluid and the tissues dissected from diseased larvae demonstrated that these OBs contained multiple-embedded, membrane-bound, and rod-shaped nucleocapsids. Furthermore,

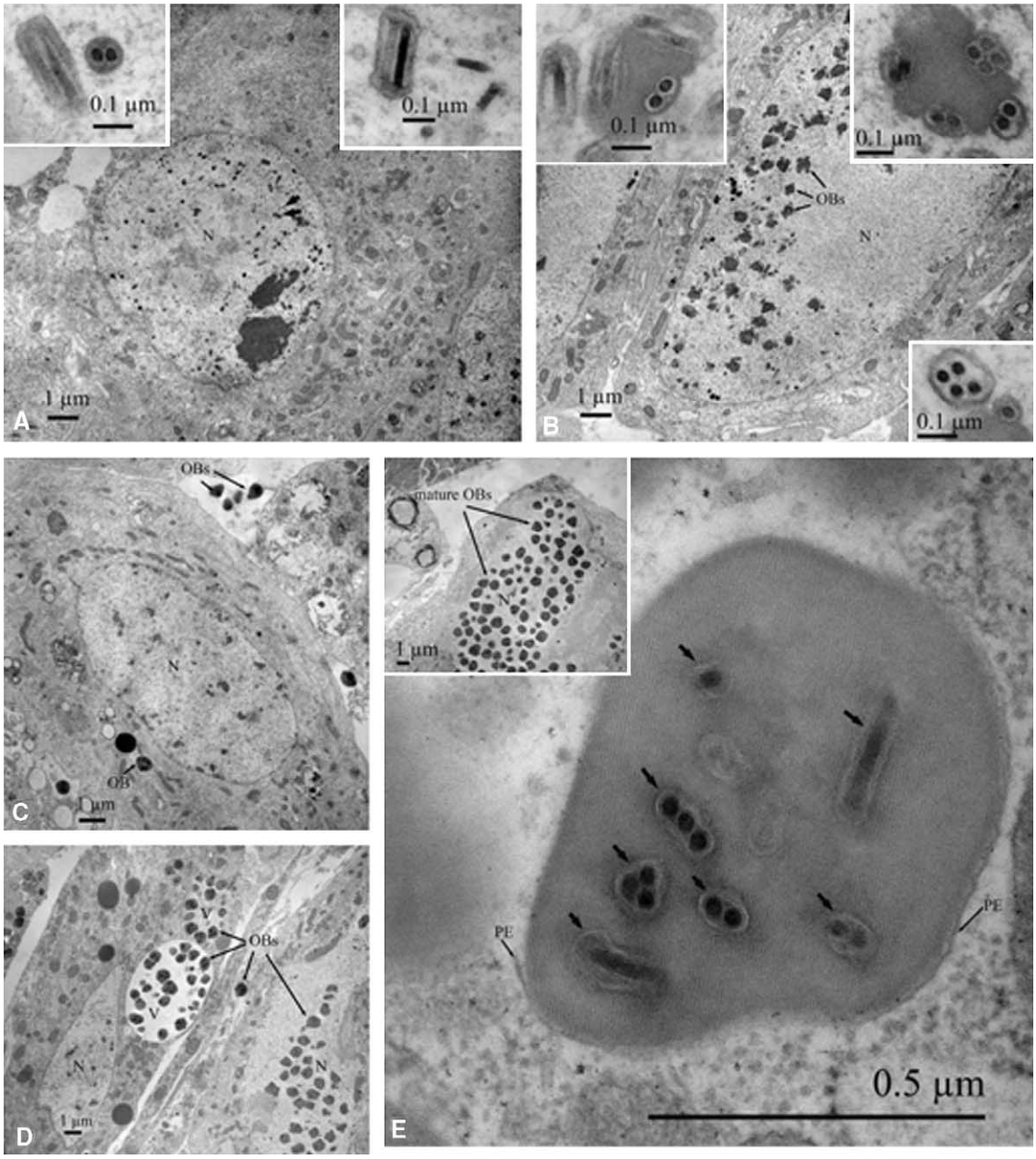

Fig. 2. Electron micrographs of MNPV infected *H. himera* cells. (A) The initial phase of virogenesis shows envelope and nonenveloped virions (arrows and insets) and no OBs in the nucleus (N). (B) The second phase of virogenesis shows the production of OBs. (C) *H. himera* cell with a noninfected nucleus showing OBs in the cytoplasm. (D) *H. himera* cell showing large cytoplasmic vesicles (V) filled with OBs. (E) MNPV infected nucleus with mature OBs (inset) and a mature OB showing polyhedron envelope (PE) and the crystalline protein lattice surrounding the bundles of virions (arrows).

electron microscopy revealed that biosynthesis of the OBs occurred in the cell nuclei (Figs. 2A and B). These observations suggested that the causal agent of the *H. himera* disease was a multiply embedded nucleopolyhedrosis virus (*Hh*MNPV). Interestingly, large numbers of mature OBs were detected in the cytoplasm (Fig. 2C) and in large cytoplasmic vesicles (Fig. 2D) of various epithelial cells. The relatively small OBs were globular in shape, uniform in size, and measured $\sim$0.7 μm in diameter. The OBs possessed the outer envelope (the polyhedron envelope of NPVs) and the crystalline protein lattice surrounding the bundles of virions that is a characteristic feature of most baculoviruses (Fig. 2E). In cross-section, each OB typically contained 3–8 rod-shaped virions and multiple (1–4) nucleocapsids that were encapsulated within the membrane of each virion (Fig. 2B). The size of the virion, including the envelope, was 50×250 nm (calculations based on single-capsid virion); the capsid size without the envelope was 25×175 nm.

Like other baculoviruses, the *H. himera* MNPV delivered per os was highly infectious to its host and lethal to challenged *H. himera* larvae. However, unlike the typical NPV, the MNPV-infected *H. himera* displayed unique disease symptoms. Virus infection did not lead to a global breakdown of tissues, and therefore, the typical liquefication affiliated with baculoviruses was not observed in *H. himera* larvae. The melting of the typical baculovirus-infected caterpillar has been heralded as a mechanism responsible for the release and dissemination of progeny occlusions (Volkman and Keddie, 1990). In fact, many baculoviruses contain genes that encode a chitinase (*chiA*) and protease (*cath*) involved in the disruption of the host cuticle (Hawtin et al., 1997). The *Hh*MNPV has adapted a slow-release strategy, and uses infected *H. himera* larvae to disseminate OBs gradually. Virus infection of the cuticle epithelium creates a weakening at the tips of the spines that results in reflex bleeding. Contact of infected larvae stimulates the release of virus-infected hemolymph through the tips of their spines, thus spreading the virus onto the plant substrate as well as depositing it on conspecifics, or to other species that are feeding on the same plant. To our knowledge this virus induced reflex bleeding has not been observed with other baculoviruses. It should be noted that Marti et al. (1987) did report that Ascovirus-infected *Spodoptera exigua* larvae did regurgitate virus discharged from the eversible gland. Furthermore they proposed that such virus discharge may contribute to the dispersal and transmission of this virus.

The origin of the disease afflicting the greenhouse colony of *H. himera* is unknown. One potential source of infection is wild *Agraulis vanillae* and *Heliconius charitonius* larvae from Florida that were reared in aquaria which were subsequently used to raise the greenhouse *H. himera* colony. Another possible source is wild local Gainesville butterflies that fed on potted nectar flowers that were later rotated into the greenhouses. Some *A. vanillae* and *H. charitonius* showed disease symptoms similar to those displayed by *H. himera*. In fact, light-microscope examination of bent spines and tissues dissected from diseased *A. vanillae* larvae revealed also the presence of OBs in the cell nuclei. Further analysis remains to be conducted with isolated virus from *A. vanillae* larvae in order to demonstrate horizontal transmission in this species.

In the case of the *H. himera*–MNPV interaction, reflex bleeding probably resulted from alteration in cuticle deposition due to the viral infection of underlying cells, weakening the tips of the spines. This, combined with an increase in hydrostatic pressure inside the body due to an increase of fluid (again caused by the viral infection, or perhaps caused by the larvae per se), produced the release of virus-contaminated hemolymph through the spines, allowing horizontal transmission of *Hh*MNPV. To our knowledge, the ability of *Hh*MNPV to induce reflex bleeding in its host insect is novel. Significantly, the *Hh*MNPV-infected insects do not undergo the normal baculovirus liquefication, suggesting that the observed reflex bleeding evolved as a substitute virus dissemination mechanism.

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References

Cuernot, L., 1896. La saigné reflexe et les moyens de defense. Arch. Zool. Exp. Gen. 4, 655–680.

Hawtin, R.E., Zarkowska, T., Arnold, K., Thomas, C.J., Gooday, G.W., King, L.A., Kuzio, J.A., Possee, R.D., 1997. Liquefaction of *Autographa californica* nucleopolyhedrovirus-infected insects is dependent on the integrity of virus-encoded chitinase and cathepsin genes. Virology 238 (2), 243–253.

Marti, O.G., Hamm, J.J., Styler, E.L., 1987. Discharge of hemolymph from the ventral eversible gland of Ascovirus-infected fall army-

worm larvae, *Spodoptera frugiperda* (Lepidoptera: Noctuidae). J. Invertebr. Pathol. 49, 127–129.
Volkman, L.E., Keddie, B.A., 1990. Nuclear polyhedrosis virus pathogenesis. Semin. Virol. 1, 249–256.

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